Stockholm 1969.

LENNART PAALZOW

Studies of analgesic and anticonvulsant activity in mice by high-frequency electrical stimulation

ACTA PHARMACEUTICA SUECICA 1969

Studies of analgesic and anticonvulsant activity in mice by high-frequency electrical stimulation

AKADEMISK AVHANDLING

SOM EFTER VEDERBÖRLIGT TILLSTÅND FÖR ERNÅENDE
AV FARMACIE DOKTORSGRAD OFFENTLIGEN FÖRSVARAS I
FARMACEUTISKA FAKULTETENS FÖRELÄSNINGSSAL,
KUNGSTENSGATAN 49, STOCKHOLM,
ONSDAGEN DEN 14 MAJ 1969, KL. 9 F. M.

AV

LENNART PAALZOW

FARM. LIC.

STOCKHOLM 1969

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Reprint from Acta Pharmaceutica Suecica 6, 227—256 (1969)

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Studies of analgesic and anticonvulsant activity in mice by high-frequency electrical stimulation[1]

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In this survey the following papers, referred to by the Roman numerals listed below, will be discussed.

I. L. Paalzow. An electrical method for estimation of analgesic activity in mice (Part I). *Acta Pharm. Suecica* 6, 193 (1969)

II. L. Paalzow. An electrical method for estimation of analgesic activity in mice (Part II). *Acta Pharm. Suecica* 6, 207 (1969)

III. S. Ljungberg, U. Otto and L. Paalzow. Analgesic activity of acetylsalicylic and salicylic acid and its relation to blood concentrations in mice. *Acta Pharm. Suecica* 5, 489 (1968)

IV. L. Paalzow. An electrical method for estimation of anticonvulsant activity in mice (Part I). *Acta Pharm. Suecica* 6, 163 (1969)

V. L. Paalzow. An electrical method for estimation of anticonvulsant activity in mice (Part II). *Acta Pharm. Suecica* 6, 177 (1969)

VI. S. Ljungberg, L. Paalzow and S. Ström. Anticonvulsant activity of ectylurea in mice. *Acta Pharm. Suecica* 5, 293 (1968)

[1] Inaugural dissertation.

VII. B. Danielsson, S. Johansson and L. Paalzow. Some new substituted hydantoins. *Acta Pharm. Suecica* **2**, 155 (1965)

VIII. H. Ferngren and L. Paalzow. High Frequency Electro-shock Seizures and their Antagonism during Postnatal Development in the Mouse I. Parameters for Electro-shock and Effect of Diphenylhydantoin. *Acta Pharmacol. Toxicol.* **27**, in press (1969)

IX. H. Ferngren and L. Paalzow. High Frequency Electro-shock Seizures and their Antagonism during Postnatal Development in the Mouse II. Effect of Phenobarbital Sodium, Mephobarbital, Trimethadione, Dimethadione, Ethosuximide and Acetazolamide. *Acta Pharmacol. Toxicol.* **27**, in press (1969)

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I. Introduction

> I often say that when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of Science whatever the matter may be.
>
> *Lord Kelvin*

It is difficult to give a fully satisfactory definition of pain. Many attempts have been made and Beecher [1], who has discussed some of them, says: "The fact is, pain is defined introspectively by every man. The difficulty comes in verbalizing this well-known experience, not very difficult in terms or statements of its presence or absence in various degrees or kinds but in saying *what* it is", and continues: "pain is measured in terms of its relief". Thus, with this limited scope in man it is obvious that it must be still more difficult to evaluate pain and its suppression in animals.

There has been considerable discussions about the "adequate stimulus" for producing pain in man and animals. Sherrington [2] introduced the term "nociceptors" for the pain receptors and he proposed that the adequate stimulus for pain is injury or nociception. This proposition has been criticized [1, 3, 4], and Lim and coworkers [4] have put forward the hypothesis that the pain receptors are chemosensitive, *i. e.* chemoceptive, and not nociceptive. A variety of chemical agents such as some amines, peptides and even H^+ and K^+ can excite the "pain receptor". They have suggested that the nonspecific character of the chemoceptor may depend upon an electrophilic attraction, assuming that the receptor sites are negatively charged or electron-rich. Winter [5] concludes in a review that "the idea of a peripheral chemical mediator for pain has much to commend it, but it has not been proven that there is any one unique substance which has been proven to be *the* mediator".

In the search for new potent analgesics, our knowledge of the physiology of pain and the development of better methods for testing new compounds obviously play an important role. In animals, existing analgesimetric methods will detect drugs with morphine-like activity but it is more difficult to obtain analgesic effects from non-narcotic agents. In addition drugs have been considered to be analgesic in some animal tests but later failed to be effective in man. And, conversely, potent analgetics in man such as the narcotic antagonists have shown minor or no effects in animal tests (for references see Paper I, II). This later type of analgesic has stressed the need of new animals procedures and it has furthermore been proposed that usual animal test methods such as the "tail-flick" and "hot-plate" tests have tended to hinder the search for non-

addicting strong analgesics, since the narcotic-antagonists have shown non or minor animal response [6]. It has also been suggested that these procedures appeared to be as good indicants of prediction of addiction liability as they are of clinical analgesia [7]. Although the salicylates have been used therapeutically since the end of the 19th century and are one of the most popular groups of drugs our knowledge of their mechanism of action is obscure. The difficulties encountered in obtaining analgesic effects in animals, have undoubtedly been a contributing factor to this hiatus.

Methodological problems have also been met in the animal tests of anticonvulsant drugs. The usefulness of both bromide and phenobarbital in convulsive disorders was discovered more or less by chance but the discovery of diphenylhydantoin by Putnam and Merritt [8] was a result of extensive experimental studies in animals. The success with diphenylhydantoin in the treatment of epilepsy established the value of laboratory assay techniques. During the succeeding decades, a battery of tests were developed and they have undoubtedly contributed to the progress of new antiepileptic drugs and to our understanding of convulsive disorders [9]. The main reason for the need of a "battery of tests" in the evaluation of anticonvulsant activity in animals seems to be that agents against grand mal and psychomotoric disturbances have been found to be highly effective in the prevention of electroshock seizures, while petit mal agents have failed or showed minor effect. On the contrary, these latter drugs have shown to be effective in suppressing convulsions elicited by chemical agents such as pentylentetrazol (see Paper I for reviews). Toman and Everett [10] have stressed the need of new animal procedures in the search for potential petit mal drugs as the existing methods show a poor predictability for these drugs.

Difficulties with the existing methods have become apparent in studies in animals during postnatal development. Investigations of the effect of anticonvulsants in immature animals have usually omitted the early neonatal period, as it has not been possible to elicit electrical seizures in intact animals during this period (for references see Paper VIII). Since the work of development has furnished important contributions to the knowledge of the anatomy and physiology of the central nervous system [11], new animal procedures might very well favour future progress. Furthermore, as seizures are not uncommon in childhood, experimental studies on seizure susceptibility and antagonism during maturation are of practical as well as intrinsic interest.

Electrical stimulation has been used for inducing both pain reactions and seizures in animals. Beecher [1] and Winter [5] have reviewed the electrical analgesimetric methods and concluded, in agreement with Hill *et al.* [12], that electrical shock is easy to apply but difficult to control, and these methods have therefore given rise to many inconsistencies. Concerning the electroshock methods in the studies of anticonvulsant activity, high voltages or ampéres are needed to produce a maximal seizure and it has been found to be unsuitable to repeat this seizure in

one and the same animal [9, 13]. This implies that an ED_{50} of a potential anticonvulsant drug had to be carried out in two stages; *i. e.* the peak-activity of the drug must be determined with one group of animals, after which the ED_{50} determination is carried out at this peak-effect with another group. The commonly used electroshock methods for adult animals are not well suited for studies during early postnatal development. The methodological difficulties in eliciting seizures in newborn animals can be overcome by spinal cord stimulation [14] or by the use of chemical agents [15, 16] but the results are very difficult to interpret.

Investigations dealing with suitable electrical parameters (frequency, pulse-width, duration of shock, etc.) for stimulation with square waves in order to produce pain or seizures in animals, have only to some extent been published. Thus, using bucco-dental electrodes in guineapigs Radouco-Thomas *et al.* [17] found certain parameters to be suitable for producing a pain response.

Woodbury and Swinyard [18] investigated the stimulus parameters for electroshock seizures in rats and found *e. g.* that any frequency in the range of 60—300 p/sec (sine-wave or square wave) would be satisfactory.

The *aim* of the present studies has been to investigate whether a carefully standardized electrical square wave stimulation could be utilized for, and perhaps open new possibilities in, studying quantitatively analgesic and anticonvulsant activity in mice. The purpose was also to investigate if it were possible to induce seizures in intact mice from the neonatal period and see if electroshock seizures in these animals could be modified by anticonvulsants.

The present investigations of an analgesic method, based upon electrical stimulation of the mouse tail, started with the examination of some factors which might possibly influence the normal pain threshold (I). The reliability and sensitivity of the method was tested by studying 10 well-known analgesics (II) and the method was then also used for more detailed studies of the analgesic effects of salicylates (II, III). Pentazocine — a narcotic antagonist that has recently been introduced in Sweden — was also included (II).

The investigations of the electrical method for estimating anticonvulsant activity in adult mice commenced with some studies of factors which might possibly influence the eliciting of maximal seizure (tonic hindlimb extension) (IV). The reliability and the sensitivity of the method developed was examined by studying 11 well-known antiepileptic drugs (V). Furthermore, the method was employed in studies of ectylurea (V, VI) and of some potential anticonvulsants (V, VII).

After methodological investigations of the possibility of eliciting seizures in mice during postnatal development (VIII), the elaborated procedure was applied in studies of some anticonvulsants in immature animals (IX).

II. Materials

A. Experimental animals

Male adult albino mice of strain N.M.R.I. normally weighing within the range 17—25 g were used in the studies of analgesic activity (I, II, III) and in the investigations of anticonvulsant effects in adult mice (IV, V, VI, VII). The animals were given a standard diet and water *ad libitum*. In the investigations of analgesic activity and in all toxicity determinations the animals were deprived of food but not of water for 16 hours before each test, but in the tests of anticonvulsant activity food was withdrawn only during the actual electroshock procedures.

In order to follow the postnatal development in mice certain ages were chosen: 1-, 3-, 5-, 9-, 12-, 15-, 18- and 21-day-old mice. Mice of the N.M.R.I. strain were used and on the day after birth they were received together with their mothers from a local breeder and then kept with their mothers until the experiments were performed. No attempt was made to separate the sexes.

The ages of the immature animals were selected on the basis of the present knowledge of the anatomical and physiological maturation [19, 20]. The weights of the mice at different ages have been published by Ferngren [15].

All experiments were performed at a temperature of 20—25°C, and were carried out in the day-time.

B. Apparatus

For the electrical stimulation of the animals, two stimulators coupled in train have been used; these supply a burst of electrical square waves, for which all parameters (frequency, pulse-width, duration of shock, voltages and polarity) can be varied. The definitions of these parameters are given in Papers I, III and IV, in addition to detailed information concerning the apparatus and the electrode technique.

C. Statistical methods

The statistical methods used were taken from Bliss [21], Burn *et al.* [22], Finney [23], Fisher and Yates [24], Gaddum [25], Ljungberg [26] and Pharmacopoea Nordica, Vol. IV [27]. The symbols used are identical with those in Pharmacopoea Nordica and those described by Ljungberg [26].

III. Studies of analgesic activity in mice by high-frequency electrical stimulation of the tail

A. Survey of the literature

Pain can be evoked by many kinds of stimuli: thermal, electrical, mechanical and chemical, and the methods used for studying analgesic drugs are usually classified according to the type of stimulus used. Consequently, a very large number of laboratory tests have been used (for reviews see ref. cited in Paper I) and, in order to be able to restrict the survey, the following discussion will be confined to some electrical methods in mice.

The use of an electrical stimulation to produce pain was originally described as early as 1851 by von Helmholtz [28]. Since then, many variations of the technique have been used in several species of animals. J. F. Reinhard and E. J. de Beer devised a method in mice but as they were not satisfied with the reproducibility of the results, the study was not formally published. However, Burn *et al.* [22] thought sufficiently well of the technique to report it in their textbook, "Biological Standardization". The squeak of the animal was taken as a response to the stimulus applied to the tail. The number of shocks required to cause a squeak was registered. If the animal did not squeak after five consecutive shocks, this animal was eliminated from the evaluation procedure. The technique was modified by Grewal [29] who, instead of assessing the degree of analgesia in each mouse, ascertained the percentage of mice showing analgesia. This method has been used and further modified by Cugurra [30], Lockett and Davis [31], Maxwell *et al.* [32], Lund Nilsen [33] and McKenzie and Beechey [34]. The most important variation, performed by Lund Nilsen [33], seems to be the predetermination of the normal pain threshold for each mouse before the test of analgesic activity. Earlier, alternating current (A. C.) had been used but the above author also introduced the square wave stimulus method. However, the only data reported concerning the electrical parameters were that the stimuli lasted for 20 msec, and were given at intervals of one per second and that the animals needed a voltage range of 2 – 64 V. Mice failing to respond by squeaking to four consecutive stimuli, when tested at the same voltage as for the pretreatment threshold, were defined as showing a "positive analgesic reaction". In the studies of analgesic drugs a great number of animals were used, *i. e.* between 8 – 1267 mice at each dose level. Also Grewal [29] used a large number of animals, whereas McKenzie and Beechey [34] only needed dose-groups not exceeding 7 mice. A modification of the latter authors' procedure has been employed by Neal and Robson [35] in order to study analgesic effects of anaesthetics given in subanaesthetic doses. Weiss and Laties [36] used another mode of electrical stimulation. Their method utilized a titration schedule in which animals are trained to depress a lever in order to reduce the current intensity of the electrical shock applied to the floor of the test cage.

B. Own studies of the influence of some factors on the normal pain threshold (I)

1. Choice of stimulus response

In a method suitable for screening potential analgesic drugs, it is necessary to register a sign of "pain" which could easily be recognized. The squeak response was chosen as a sign of "pain" as this response seems to give advantages. Thus, the squeak response to a noxious stimulation is considered by Guzman *et al.* [37] to be the most specific indicator of pain in animals, and McKenzie and Beechey [34] proposed that the vocal response offered many advantages and could perhaps be regarded as analogous to vocal components of human reactions to pain.

Vocalization disappears after midbrain section [5] but persists after transection at a lower pontine level, suggesting that the reflex pathway is through the medulla oblongata [38]. Hoffmeister and Kroneberg [39] have therefore proposed that the vocalization afterdischarge also should be investigated in order to study the integrity of a thalamo-hypothalamorhinencephalic system. This system is thought to be associated with the capacity for affective reaction. Such studies are, however, circumstantial and not well adapted for screening purposes.

Reflexes that have often been taken as indications of a noxious stimulation in animals are the tail flick, the back skin twitch, the lifting of the hind leg from the hot plate or the squeak response. Benjamin [40] regarded the usual endpoints used in animals *e. g.* skin twitch or tail-flick — as side effects of pain since they are spinal reflexes.

In the present investigations, it was observed that reflex movements of the tail of the animal were found at a lower voltage than that needed to produce a vocalization. This further emphasized that the squeak as a response involves the participation of higher centres in the central nervous system. This stimulus has also been applied to man in the same manner as for mice (I), except that the electrodes were placed intracutaneously at the ulnar edge of the volar side of the hand. With a voltage of the same magnitude as used in mice, a distinct painful stimuli was experienced [41]. This could indicate that the pain reaction was of the same type as in mice. It has, of course, been discussed whether an animal feels pain, and Beecher [1] has expressed: "Pain is pain to an animal, presumably, and all pain serious and significant".

2. Electrical factors and the reproducibility of pain threshold

In the present investigations, the *pain threshold* was defined as the minimum voltage necessary to induce a pain reaction (mean of three single determinations). Such reaction was regarded to be obtained when the animal responded to one of the first three consecutive shocks by squeaking. The shocks were applied at intervals of one second through intracutaneously applied electrodes in the middle part of the tail.

In order to elicit the most suitable electrical parameters for inducing a squeak response in the animals, studies were made of the importance of frequency, pulse-width, duration of stimulus and the voltage of the square waves for the production of a pain response. The number of animals showing a pain reaction were increased with the increase of these parameters until a plateau was reached. The indices of each parameter for maximal response were adopted as the suitable stimulus parameters, *i. e.* frequency 125 p/sec, pulse-width 1.6 msec and duration shock 40 msec. It was interesting to note that the high frequency chosen was of the same magnitude as the reported frequency discharge in pain fibers [42, 43]. The fact that certain frequency indices give rise to more pain-reacting animals than other indices was in accordance with Weddel [44], who suggested that pain is a warning signal depending solely upon the number of action potentials from free nerve endings entering the spinal cord for analysis and interpretation in the central nervous system. Furthermore, about the same index as the actual adopted frequency has been found by Hensel and Boman [45] in recordings from non-specific fibers during cutaneous mechanical and thermal stimulation in man. Using bucco-dental electrodes in guinea-pigs, found Radouco-Thomas *et al.* [17] that 40 p/sec and a pulse-width of 5 msec were suitable for a shock applied during 0.5 sec.

The electrodes were applied intracutaneously and kept in position during each experiment. The polarity must be fixed, and it was found that the most sensitive positions were obtained when the positive pole was applied proximally. It was assumed that intracutaneous application would minimize the error due to changes in skin impedance. In the methods described by Burn *et al.* [22], Grewal [29], Maxwell *et al.* [32] and McKenzie and Beechey [34], the electrodes were applied on the skin surface of the tail, which had first been cleaned with ether, and fixed with conductive paste in order to provide reproducible results. If the intracutaneous electrodes used here had to be removed after an experiment and were later again applied to the same animal, the possible variation in tissue impedance was eliminated by the fact that the individual pain threshold for each mouse was taken into account.

The pain threshold was determined in three pretests and the mean voltage was taken as the definite value. As has been pointed out in Paper I, considerable interest has been paid in the relevant literature to the question, of whether of a produced constant voltage, current or power (wattage) is the best index of sensory effects. In the method used here, the import of this problem is less acute as the individual pain threshold for each mouse is taken into account. The variations in pain threshold within the individual animal can be found in the investigations of the placebo effects (graded response) or the number of placebo reactors. The pain thresholds after placebo were found to be quite stable and within a range of 95– 110 %, where 100 % corresponds to the pain threshold found in the pretest (Figs. 9, 10 and Table 1 in Paper I). In the quantal

response studies the number of placebo reactors on separate occasions during a test were up to 27 % if the predetermined pain threshold voltage was used (Fig. 7 in I). The percentage of placebo reactors could be reduced to less than 5 % when a 25 % increase of the threshold dose was given. This was considered as a statistically satisfactory low number of placebo reactors and was also obtained from day to day (Fig. 8 in I). The appearance of a placebo reactor occurred quite at random and was consequently not typical for a certain animal. The appearance of placebo reactors could of course just as well be a consequence of the unknown biological variation as a change in skin impedance or any other physical factor. As these factors are presumedly randomized, any bias of the results found could be neglected.

A collocation of different pain thresholds for 1000 mice showed that 95 % of the animals had thresholds of less than 11.1 volts (10.9 – 11.3 volts at $P = 0.05$).

The limited range of the pain threshold voltages and the satisfactory small number of placebo reactors from day to day could perhaps be utilized for screening potential analgesics in a test where all animals receive the same voltage.

3. Weight or ageing

In the present investigations of the pain threshold as a function of body weight in mice (Fig. 5 in I), the sensitivity seemed to decrease with increasing weight. However, it should be noted that, in parallel with the weight increase, the mice grew older but no account was taken of which of these two factors was responsible for the pain threshold change. In the analgesic studies the importance of different sensitivity at different weights is diminished by the fact that the individual pain thresholds for each animal are considered.

Beecher [1] has drawn attention to the fact that both pain perception and pain reaction are found to decrease with increasing age.

4. Conclusions

The method introduced implies that a "pain reaction" is produced by a high-frequency electrical stimulation of the tail via intracutaneous electrodes and to which the animal responds with a squeak.

A stable pain threshold was obtained as a result of standardization of the parameters of the electrical square wave stimulus (frequency, pulse-width, duration shock, polarity) to certain maximum values for producing a pain response and, further, by taking into account the individual pain threshold of the animals. The pain threshold remains sufficiently constant for the time needed to test a potential analgesic drug, even when the test is repeated on a consecutive day (cross-over test).

C. Application of the method in investigations of some analgesic drugs (II, III)

Animals which, after administration of a drug, did not respond with a squeak to three consecutive stimulations of the 25 % increased threshold voltage, were considered to show an analgesic effect.

1. Importance of the individual sensitivity (pain threshold voltage) for the analgesic effects obtained

In my investigations, no relation between magnitude of the pain thresholds and the effects obtained from several analgesics could be found (Fig. 5 in II). This may indicate that the increase of the voltage in logarithmical steps by the determination of pain thresholds is a suitable procedure. Other authors [46, 47] have found it preferable to use only animals with high sensitivity in investigations of analgesic drugs and Lund Nilsen [33] has found that animals with the lowest pain threshold voltage were more sensitive to the effect of codeine.

2. Potencies of well-known analgesics, quantal response

The quantal analgesic effects of 10 well-known drugs have been studied and rectilinear log dose-response lines were obtained. In order to ensure that no toxic effect had merged with the analgesic effects, preliminary calculations of the acute toxicity (LD_{50}) were performed. The following drugs were investigated: Acetylsalicylic acid, aminophenazone, codeine, methadone, morphine, pethidine, phenacetin, phenazone, dextropropoxiphene and sodium salicylate.

In general, the *morphinomimetic drugs* showed a potency in accordance with that found in other laboratories and with similar or other methods (reviews cited in Paper I and II). The slopes of regression lines in the ED_{50} determinations of metadone, morphine, pethidine and codeine did not differ significantly ($P > 0.05$) from each other, but dextropropoxiphene showed a significantly steeper slope ($P < 0.001$) (Table 3 in II). An explanation of this is difficult to find. Morphine and related drugs showed a markedly higher effectiveness when administered parenterally than when given by the oral route and the probable reason for this was discussed in Paper II. It seemed reasonable to assume that the lower effectiveness of oral route administration could be explained by physicochemical factors and the degree of biotransformation in the liver. Morphine is conjungated in the liver to glucuronic acid and this conjungation is found in rats to be an inactivation process [48, 49]. Jóhannesson [50] has found that codeine, in contrast to morphine, had a stronger effect in rats when given by intraperitoneal injection than after subcutaneous administration and he proposed that this difference might be a consequence of a lesser degree of inactivation in the liver of codeine than of morphine. This proposition might perhaps be valid for mouse in the present investigation with respect to the oral *versus* the subcutaneous

effectiveness. Codeine was furthermore found to be more effective (equimolecular comparison) than pethidine by the oral route but showed a somewhat lower therapeutical index (Table 2 in II).

The *weak analgesics;* aminophenazone, phenacetin, phenazone, acetylsalicylic and salicylic acids, all showed effects in doses at a safe distance from toxic dose levels. Aminophenazone was the most potent while the other four gave confidence limits overlapping each other. Phenacetin had a somewhat higher therapeutical index than the others.

In general, the analgesic effect of the weak analgesics in animals is more difficult to demonstrate than their antipyretic effect. Randall [51] has stated that this is due to the lack of sensitive methods. However, he points out that, generally, acetanilide, acetophenetidin (phenacetin) and N-acetyl-p-aminophenol (NAPA) are held to be analgesics in animals. Kadatz [52] found that in mice exposed to the "hot plate" technique, phenacetin was effective at a dose level of 181 mg/kg and Lund Nilsen [33] reported an $ED_{50} = 270$ (210 -420) mg/kg after intraperitoneal injection. Using rats, Boréus and Sandberg [53] found that phenacetin was more active than its chief metabolite NAPA.

Phenazone (antipyrine) has mostly failed to show analgesic effects in animals except at toxic dose levels [51], but against chemically induced "writhing" syndrome phenazone was found to be effective in mice [54, 55]. However, this test, which is commonly used, has been reported to lack specificity since a large number of non-analgesic drugs are found to give a favourable effect [51, 54, 55]. The pharmacology of phenazone has been reviewed by Greenberg [56] who concludes that our knowledge of the analgesic effects is mainly derived from clinical experience. Numerous reports on the effectiveness in man, using different experimental stimuli, have been published, but there is no agreement as to its analgesic activity.

Woodbury [57] suggests that the antipyretic and analgesic mechanism of aminophenazone and phenazone is similar to that of the salicylates. In the present investigation the analgesic effects in mice were rapid in onset in contrast to the salicylates which showed the slowest onset of all investigated drugs. This could be explained by the physico-chemical properties of the drugs as phenazone has a higher lipid to water partition coefficient than salicylic acid at pH — 7.4 [58]. Consequently, the non-ionized phenazone has been reported by Brodie [58] to enter canine cerebrospinal fluid rapidly in contrast to salicylic acid. The absorption of phenazone in rats is found to be good, but less than that of salicylic acid [59]. The same type of difference in physico-chemical factors could perhaps explain the shorter time for the onset of analgesic effects of aminophenazone than for that of salicylic acid.

3. Graded changes of the pain threshold

The method introduced offers two possibilities of studying analgesic effect, *i. e.* either the quantal response or the graded changes in the pain threshold. The former procedure has the advantage of being more rapid

in performance as the pain threshold need not be established for each test occasion. The graded changes of the pain threshold were expressed as the threshold voltage found in percentage of pain threshold voltage found in the pretest.

Thus, as a comparison, the graded changes of the pain threshold have been investigated after oral administration of methadone, pethidine and phenazone. The same doses as those used in the quantal response were chosen and rectilinear log dose-reponse lines were found. Tentative information concerning the sensitivity of the quantal method could be obtained by studing the elevation of the pain threshold with the found mean effective dose. Thus, the quantal method seemed to be more sensitive as the ED_{50}-doses of these three drugs caused a rise in the pain threshold of about 30—50 %.

The graded changes after application of morphine and pentazocine intravenously have also been studied and will be discussed later.

4. Acetylsalicylic and salicylic acids

In the present investigations both acetylsalicylic acid and salicylic acid were found to be effective, applied orally as solutions or suspensions. The salicylic acid consistently produced a quicker effect, which might have been a consequence of more rapid absorption. This was also apparent from the blood analyses which showed that sodium salicylate gave higher blood levels more rapidly than did the corresponding soluble calcium salt of acetylsalicylic acid (Figs. 5 and 7 in III). Even when the soluble salts of acetylsalicylic and salicylic acids, respectively, were given intravenously the latter acid showed a more rapid onset of analgesic effect and there was also a delay in effects from acetylsalicylic acid which may be partly due to the conversion of this acid to salicylic acid. Although the salicylates have been used therapeutically since the end of the 19th century, it has not been possible to correlate their analgesic effect to the concentrations in plasma or other tissues. The probable reasons for this failure are discussed in Paper III. Lester *et al.* [60] draw attention to the fact that acetylsalicylic acid in itself may act as an analgesic *per se*, and not only by the salicylic acid fraction formed by hydrolysis. Other factors such as the lack of sensitive methods for evaluation of the effects and the importance of protein binding might have contributed towards the lack of correlation between effect and content in the body.

In the present investigation it was found that it is important to estimate the blood content of "free" salicylic acid and not only the content of total salicylates. It was also found that the presence of unhydrolyzed acetylsalicylic acid in blood did not give rise to any analgesic effect which could be ascribed to the acid as such. However, a good correlation was obtained between the blood content of salicylic acid and effect. It is therefore probable that acetylsalicylic acid exerts its effect through the hydrolytic product, salicylic acid. If the unhydrolyzed acetylsalicylic acid were the agent which exerts the analgesic effect at the site of action it is not

unreasonable to expect adequate concentrations in the blood coincident with the effects obtained. However, in the mouse the hydrolysis of acetylsalicylic acid was found to be very rapid and at the time for maximal analgesic effect the content of the unhydrolyzed acid in blood was not detectable or could be neglected.

Another side of the problem is actually the site of action. The effectiveness of salicylates in relieving pain associated with inflammation, has often been taken as evidence of a peripheral site of action, but acetylsalicylic acid and related drugs are generally held to have effects beyond the anti-inflammatory [5]. The antipyretic effect occuring together with the analgesic activity, has often been taken as an indication of a central site of action and the salicylates should therefore affect the hypothalamus. However, there is evidence that acetylsalicylic acid may have antipyretic effects which are independent of the thermoregulatory centre in the hypothalamus [51]. Paper III offers a discussion on whether the salicylates exert their analgesic action at a central or a peripheral site. Although it has not yet been proven, it seems reasonable to assume, on the basis of these arguments, that the salicylates have an analgesic activity which is a combination of central and peripheral effects. Concerning the unhydrolyzed acetylsalicylic acid, this substance has not yet been detected in the brain [4] and therefore it seems unlikely to expect this acid to possess central activity.

In the present investigations of the correlation of the analgesic effects and the content in blood, it was found that animals showing effect had higher levels of salicylic acid than animals not showing any effect but no statistically established difference was obtained (III). If, instead, the brain content of salicylates was determined at the time for maximal effects, a significantly higher mean level was found in the animals showing effect than in the animals not showing an effect (Table 10 in II). Thus, it seems reasonable to support Winter's opinion [5] that the salicylates have a central site of action besides a peripheral one.

The slow onset of the analgesic effects found in mice could be explained by a slow penetration to brain tissues. At the actual pH of blood, salicylic acid is present mainly in the ionized form and the rate of entry to the central nervous system is dependent on the available amounts of the non-ionized form; this is based on the assumption that the penetration through biological membranes is a passive diffusion [58, 61]. The slower onset of analgesic effect of acetylsalicylic acid compared to salicylic acid given intravenously (Fig. 4 in III) could be explained by the time needed for the former acid to be converted to salicylic acid.

The ceilings of the analgesic effects found could also probably be explained by a slow passage to brain. If the doses of the two salicylates investigated are increased over 400– 500 mg/kg a levelling-off of effects was found and no further increase of effects was noted until toxic symptoms appeared. Preliminary experiments have indicated that if the blood concentrations of salicylic acid can be maintained at a sufficiently high level during an adequate period of time, the ceiling effect could be overcome [62].

The analgesic effects obtained in mice could of course be partly explained as an anti-inflammatory or an anti-preinflammatory action of the salicylates, but it would be difficult to explain why the salicylates in the present investigations raised the pain threshold.

As a conclusión drawn from the results obtained here in mice, it is proposed that the salicylates have a central site of action for analgesia apart from other effects. The recently reported dose-dependent effect of acetylsalicylic acid on human EEG supports this assumption [63], but the results are in contradiction to the opinion offered by Lim [4] that the salicylates do not act centrally in analgesia. Irrespective of the mechanism of action, it is of fundamental importance for the magnitude of the effect to know how great a part of the salicylates is bound to the albumin, as only the free part has effect. However, only a slight degree of albumin binding has been found in the mouse as opposed to man [64].

5. Pentazocine

Pentazocine is a narcotic antagonist which has shown no or minor animal response in the "tail-flick" and "hot-plate" technique [65]. The effectiveness is well established clinically (references cited in Paper II) in doses twice to four times that of morphine (by weight).

Solutions of pentazocine lactate showed, in my investigations, a ceiling quantal response at 40—50 % of the animals regardless of route of administration.

Ljungberg [66] has introduced a method for intravenous application of drugs not soluble in water by preparing emulsions of refined soya bean oil with water and with the drug dissolved in the oil. The placebo effects were found to be within the range for physiological saline solution. The fat globules could be compared to chylomicrons normally found in blood after digestion of fat.

Pentazocine given intravenously in this manner gave no detectable ceiling in the same doses as the soluble salt and an $ED_{50} = 7.4$ (5.9—9.6) mg/kg was obtained. The placebo-effects obtained in the test of analgesic activity were in the same range for the emulsions as for physiological saline solution. The ED_{50} of morphine in a comparative test was 4.2 (3.9 -4.7) mg/kg. A rapid biotransformation of pentazocine in mice could perhaps explain why this drug has failed to be effective in commonly used tests. By preserving the drug in fat globulus, the active drug could partly be withheld from biotransformation. The metabolic pathways in mice are not known but my own preliminary studies [67] have supported this assumption. The effects were rapid in onset and the graded rise in the pain threshold was rectilinear with a significant slope ($P < 0.02$). The slope differed significantly ($P < 0.001$) from that of morphine. No detectable toxic effects were observed either with the water solutions or with the emulsions given intravenously. Recent subsequent determinations of the acute toxicity of the two preparations have shown no statistically significant difference, with the LD_{50} values in the dose range of 24—29 mg/kg [67].

6. Non-analgesic drugs

The sensitivity and reliability of the method introduced for the estimation of analgesic activity in mice were investigated by studying well-known analgesics, but some newly synthesized quinazolone derivatives were used [68] to penetrate the problem of the specificity of analgesic effect. None of these was found to have any analgesic effect even in subtoxic doses.

A pharmacological effect, such as sedation, might perhaps interact with the squeak response. In mice, analgesic agents such as morphine and phenazone have been found by Ström [69] to give a significant reduction of the spontaneous activity. It was therefore of interest to study a non-analgesic sedative drug with the method introduced. Phenobarbital was chosen as it has been found in our laboratories by Ljungberg [70] and Ström [69] to induce a significant reduction of the spontaneous activity in mice within a dose range of 10 – 80 mg/kg both subcutaneously and orally. However, no reproducible influence of the pain threshold was found in these doses by the oral route.

7. Correlation to clinical potency

As pain includes several physiological and psychological processes in man and as we are unable to comprehend how an animal experiences pain stimulus, an extrapolation must be regarded as a speculation. However, Beecher [1] has pointed out that "there has been fairly general agreement up to the present time that the reaction threshold changes (it is not permissible to call this pain threshold) produced in animals by analgesic agents are often useful in appraising analgesic power".

An attempt was made to relate the doses used clinically to the mean effective doses found in the present investigations. Only the potencies of some morphine-related drugs were compared, largely because of conflicting reports on the adequate doses and potencies in man of weak analgesics. Houde *et al.* [71] have drawn attention to the aspect that, in spite of a fairly voluminous literature, there are few reports of the relative activity of these latter drugs. With regard to acetylsalicylic acid, they pointed out that there have been no reports of the relative analgesic potency compared to a "standard analgesic" in assays where graded doses of both drugs were used.

However, it can be seen from Table 9 in Paper II that there appeared to be good agreement in the relative potencies between the findings in animals and in man. Pethidine and codeine were perhaps somewhat more effective in mice compared to morphine. Pentazocine in mice was found to be about half as active as morphine (equimolecular doses) and this agrees well with the clinical experiences (references cited in Paper II).

8. Conclusions

The reliability and sensitivity of the analgesimetric electrical method introduced have been examined by testing 10 well-known analgesics. Both

the morphinomimetic and the antipyretic drugs have shown significant rectilinear log dose-response lines (ED_{50}) in dose levels at a safe distance from toxic doses (LD_{50}). Even the graded changes of pain threshold after administration of methadone, morphine, pethidine and phenazone have been studied and were found to give rectilinear log dose-response lines. The method is well adapted for screening technique.

The sedative effects of phenobarbital did not merge with the pain response.

The possibilities of studying analgesic effects of the salicylates thereby opened have been utilized for investigations of the correlations of effect to blood and brain concentrations. On the basis of the results obtained it is proposed that, apart from other effects which could influence the analgesia, the salicylates (*i. e.* salicylic acid) have a central site of action for the analgesic effects in mice. Moreover, acetylsalicylic acid seems to exert its effect after conversion to its hydrolytic product, salicylic acid. A good correlation was found between the analgesic effects and the content in blood of salicylic acid and a significant correlation to the content in brain of salicylates. A theory of ceiling effect of the salicylates is proposed *i. e.* a slow penetration rate through the blood-brain barrier.

Pentazocine has here shown a significant rectilinear dose-effect curve and the earlier reported lack of effectiveness in small animals might be explained by a rapid biotransformation.

A relatively good agreement between the relative effective doses in man and in mice was found for morphinerelated drugs.

IV. Studies of anticonvulsant activity in adult and neonatal mice by high-frequency electrical stimulation

A. Survey of the literature

Convulsions are induced experimentally by several different stimuli. Mostly used today are methods where seizures are provoked by electrical shock, chemical agents, hyperthermia, sensory stimuli or chronically upheld epileptogenic lesions. In adult mice the procedures mainly used are seizures provoked by supramaximal or maximal, minimal and low-frequency electroshock stimulation (for reviews see Paper IV).

The minimal electroshock seizures have been referred to as threshold seizures. They consist of clonus of the face, lower jaw or forelimbs, without loss of upright posture [9]. If the A. C. current needed to produce these seizures (about 6– 9 mA in mice) is increased about 5 – 10 times, a maximal or supramaximal seizures are provoked. The convulsive pattern obtained with such stimulus can be divided into three components 1) a tonic flexor phase, 2) a tonic extensor phase and 3) a clonic phase. These seizures are followed by a post-convulsant depression and often death. In the supramaximal or maximal tests the animals are used only once. The electrical shock dose is about 50 – 150 volts A. C. (50 – 150 mA)

and mostly shock of a frequency of 50– 60 p/sec is delivered during 0.2 or 0.3 sec through corneal electrodes [72]. Toman and coworkers [10] have used square waves of one msec pulse-width and with a frequency of 100 p/sec. A voltage of 140 V amplitude for the shock delivered during 0.3 msec are used in eliciting a supramaximal seizure in mice.

The most common electroshock procedures for induction of convulsions in adult animals are not well suited for studies in *immature animals*. With the electroshock procedure introduced by Woodbury and Davenport [73], Millichap [74] could not elicit tonic or symmetrical clonic seizures in rats below the age of 10 days. Vernadakis and Woodbury [75] were able to elicit seizures in mice and rats older than 8 days with A. C. current of 60 p/sec and they were able to demonstrate an anticonvulsive effect of phenytoin. Vernadakis and Woodbury also found threshold convulsions in mice from the age of 13 days with the low-frequency electroshock procedure introduced by Toman [76]. Esplin and Freston [77] introduced the technique of spinal cord stimulation with square wave stimuli and showed that their procedure gave the same seizure pattern in decapitated adult animals as maximal electroshock seizures seen in intact animals. Their procedure was used and modified by Vernadakis [14] for rats during postnatal development. In decapitated rats, this author could elicit seizures even in one-day-old animals with frequencies between 25 and 300 p/sec with a duration of the stimulus of 10 seconds. Ferngren [15, 16] found that the methodological difficulties in eliciting seizures in animals during the early neonatal period could be overcome by the use of chemical convulsants, but the requirement of a new method was stressed if the development of the seizure pattern and if the duration of anticonvulsant effect were to be followed in one and the same animal.

B. Own studies of the influence of some factors on the normal seizure susceptibility (IV, VIII, IX)

1. Choice of stimulus response

As the determinations in adult animals were of the type "all-or-none response" it was essential to have an easily assessable effect limit. *Seizure* or *convulsion* in these animals were defined as an attack with tonic *hindlimb* extension.

As the tonic hindlimb seizure did not appear to a sufficient extent until an age of 18—21 days in the immature mice, the duration of tonic *forelimb* extension was registered.

2. Electrical factors and the reproducibility of the seizures

In order to deduce the most suitable electrical parameters for inducing seizures in the animals, studies were made of the wave-form, frequency, pulse-width, duration of stimulus, and the amplitude (voltage) of the square wave. It was found that an intermittent square wave was more

suitable than a D. C. current (Table 1 in IV) and, as was the case with the parameters for inducing a pain-reaction, an increasing number of animals showed convulsions with the increase of these parameters until a plateau was reached or certain indices attained maximum. It was found that in adult mice a frequency of 800 p/sec and a pulse-width of 0.6 msec proved to be most suitable for shock administered during 400 msec. The seizure susceptibility was maximal at higher frequencies than the pain stimulus response. This high frequency is in accordance with the frequency discharge found in human epileptic foci whereas the pain stimulus frequency discharge was more connected with the activity in pain fibers.

By using these electrical parameters and a certain fixed position of the temporally applied electrodes it was possible to repeat the tonic hindlimb extension every 15 minutes in each animal without the type of response being changed. The voltage needed to produce the seizure was determined for each mouse in two pretests and was called the *convulsion-threshold*. In a collocation of 716 animals (Fig. 7 in IV), 95 % of them showed seizures at a voltage of 8.7 V or less, which corresponds to a current of about 5 mA and which is only 10 % of that used in earlier methods for maximal or supramaximal stimulation.

As the animals survived this low voltage stimulation it was possible to repeat the seizure and it was therefore possible to follow the effects of an anticonvulsant drug in one and the same animal. However, in order to be able to repeat the convulsions every 15 minute for 3 hours with a convulsion rate of at least 95 %, the threshold voltage had to be increased by 20 % and, if necessary, the shock given twice. Despite the report by Brown [78] that tonic hindlimb seizure could be provoked in less than one minute, this possibility has not been used in determinations of anticonvulsant activity. It has only been utilized for estimations of the time for onset of peak effects of drugs while the subsequent ED_{50} determinations were performed on other groups of animals. Several authors consider it unsuitable to repeat a tonic hindlimb seizure because a changed convulsion pattern is obtained [9, 13].

Concerning the immature animals, 1-, 3- and 5-day-old mice had a maximal incidence of forelimb seizure at a frequency of 100 p/sec, and at 800 p/sec one-day-old mice had no seizures at all (Fig. 1 in VIII). In older animals, forelimbs seizures occurred at higher frequencies, and 18- and 21-day-old mice resembled adult animals. The finding that animals in the early postnatal period respond to lower frequencies is difficult to explain. Vernadakis [14] obtained similar results with spinal cord stimulation in decapitated rats. It could be due to a physical phenomenoma since a current of low frequency goes deeper than a current of high frequency, or it could indicate that immature brain cells and structures are more easily stimulated by square waves of somewhat lower frequencies.

Regardless of age, the number of animals showing seizure (tonic forelimb extension) increased, as for the adult animals, with increasing pulse-width until a plateau was reached, this pulse-width should be about 30—60 % of the pulse-interval (Fig. 2 in VIII). The duration of shock

showed no special age-dependent characteristics and seemed more to be a measure of the current supplied. After the investigations into suitable electrical parameters for developing mice, certain indices were selected and these were generally those which had given maximal seizure susceptibility (Table 1 in VIII).

The voltage used to induce threshold seizure decreased with increasing age and, in 21-day-old mice was about the same as that found for adult animals.

The mean duration of tonic forelimb seizures from the pretests of the different age groups showed significant differences ($P < 0.01$) between adjacent age groups and even between all age groups, with certain exceptions. Thus, there were no established differences between 3-, 9- and 15-day-old animals (Table 1 in IX).

Control animals which were handled exactly as the druginjected animals, except that they were given the used solvent as placebo, often showed an increase in the duration of the forelimb extension in comparison to that found in the pretest (Fig. 13 in IX). The most marked change was observed in the 3-day-old and 5-day-old mice 30 minutes after pretest, while 9-day-old animals showed no special trend during the repeated stimulation. 15-day-old mice gave a successive increase during the whole experimental series 12-day-old animals showed the shortest duration and 1- and 21-day-old the longest (pretest values). The increase in the durations during repeated stimulation and the differences in durations between certain age-groups are rather difficult to interpret. The influence of corticosteroids on the found increase in seizure duration has been investigated by Ferngren [79] who found that the mechanism was steroid-sensitive but was not caused by steroid-release and, further, that a certain level of natural corticosteroids is necessary to produce this found increase. The found differences in duration between age-groups could perhaps be a consequence of the development balance of the inhibitory and excitatory systems (for review see Kreindler [80]).

3. Electrode technique

The most suitable positions for the intracutaneous needle electrodes were investigated by χ^2-analysis in adult animals. Positions at the edges of the ears were studied and the polarity was taken into account. Four sites and the different possibilities for placing the two electrical poles gave 28 alternatives (Table 2 in IV). The results were rather difficult to interpret, but certain regions of the brain seemed to be more sensitive to electrical stimulation than others. It was however, obvious that if constant experimental conditions were to be established the positions or the polarity, as well as the electrical parameters, must be standardized.

In the immature animals the same position was chosen as for the adult *i. e.* in the temporal region with the positive electrode medially at the edge of the left ear and with the negative electrode at the lateral edge of the right ear.

4. Seizure pattern

The duration of the tonic hindlimb extension in adult mice showed no change during the repeated stimulations (Fig. 6 in IV). Other changes were not studied since hindlimb extension only had been selected as the required response (quantal response).

On the other hand, the immature animals showed changes during development. Firstly, it was possible to elicit tonic forelimb seizure in all age groups studied. Hindlimb extension occurred in some animals from the age of 9 days and increased in frequency during development. From the age of 18 days, a seizure pattern like that in adults was seen in most mice and in all 21-day-old animals (Fig. 4 in VIII). The maturation of the electroshock seizure pattern fits with other developing processes in the brain function [19, 20].

5. Weight or ageing

The immature animals showed an increasing sensitivity to the electrical stimulation with maturation. The low excitability in the younger animals could be attributed to special membrane properties, the small surface area and the primitive dendritic tree of the immature neurons. Other factors such as the extensive extracellular space and the high water content in the young brain might also have contributed to the lower sensitivity [81].

The increasing sensitivity with increasing age or weight was observed in adult mice and was thus in contrast to the decrease found in the animal's perception of a pain stimulus. The importance of different sensitivity at different weights or ages in the animals is diminished by the fact that the individual seizure threshold of each animal is taken into account in each experiment.

6. Conclusions

An electroshock procedure utilizing high-frequency square wave stimulation has been elaborated for mice. Standardization of the parameters of the square wave (frequency, pulse-width, duration shock, polarity) to certain maximal values for producing a seizure and furthermore, consideration of the individual seizure threshold of the animals have furnished new possibilities of studying anticonvulsant activity.

Thus, the tonic hindlimb seizure in adult mice can be reproduced every 15 minutes without the type or time of response being changed and this has opened the possibility of following the duration of anticonvulsant activity in each animal. The voltage needed to produce the tonic seizure is only about 10 % of that used in methods described earlier. The frequency of the square wave showed maturation characteristics. Younger animals were found to be more sensitive for lower frequencies (100 p/sec) while adults were sensitive to higher frequencies which correspond to the discharge in human epileptic foci (800 p/sec).

It was possible to elicit tonic forelimb seizure in all the age groups studied. The seizure pattern changed with maturation and showed adult characteristics in 15-day-old animals. Thus, the possibility of studying the anticonvulsant activity during maturation was offered.

C. Application of the method in investigations of some anticonvulsant drugs (V—IX)

In the *adult animals, an anticonvulsant effect* was considered to exist when the tonic hindlimb extension failed to occur after one or two consecutive shocks of a voltage corresponding to the individual convulsive threshold voltage with a 20 % addition (quantal response).

In the *neonatal mice* the reduction in duration of the extension of forelimbs was taken as a measure of *anticonvulsant effect* (graded response). Threshold voltage for inducing the seizures was determined in each animal and used throughout the experiment, except in 18- and 21-day-old mice where the voltage were increased by 20 % during the tests in order to ensure at least 95 % convulsions.

1. Potencies of antiepileptic drugs in adult mice

The quantal response of 11 well-known antiepileptic drugs was studied after oral administration. The following drugs were investigated: Acetazolamide, carbamazepine, ethosuximide, ethotoin, phenacemide, phenobarbital sodium, phensuximide, phenytoin, primidone, sodium bromide and trimethadione.

A rectilinear relationship was found between the logarithm of the doses and the effects (probit). As in the case of the analgesics, the slopes of the regression lines showed no conformative trend (Table 2 in V resp. Table 3 in II). Nor was there any correlation between the magnitude of slope and the clinical use according to the type of epilepsy. In general, grand mal agents have earlier been found to be effective against electroshock while drugs used in the treatment of petit mal seizures have failed or shown only minor effects. On the contrary, phensuximide, used in petit mal treatment showed here the steepest slope. Thus, is was interesting to note that all antiepileptic drugs studied showed anticonvulsant effects in doses at a safe distance from toxic dose levels; this also applied to the petit mal agents. The ED_{50} values (Table 1 in V) were low throughout 10—50 % compared to those obtained from other electroshock procedures (see references cited in Paper V).

The onset of the anticonvulsant effects differed between some of the drugs studied and the presumable reasons for this have been discussed in paper V. Thus, the grand mal agents, phenytoin, phenobarbital and primidone, showed a slow onset of action and this is assumed to be a consequence of a slow absorption rate. The slow onset of effects from sodium bromide and acetazolamide were suggested more to be a consequence of another mode of action or to be caused by a slow penetration rate through the blood-brain barrier, as the absorption rates have been found to be fairly good [82]. The petit mal agents phensuximide and trimethadione showed a rapid onset of anticonvulsant effects as opposed to ethosuximide.

2. Potential anticonvulsants in adult animals

The sensitivity and reliability of the method introduced for the estimation of anticonvulsant activity in mice were investigated by studying well-known antiepileptic drugs, but in order to study the specificity of the method some newly synthesized substituted hydantoins were included (VII). Four of these showed effect while 7 compounds failed in doses up to about 25 % of LD_{50} or in doses up to 100 or 200 mg/kg. The structure-activity relationship are discussed in Paper VII. Furthermore, benzoyl-phenobarbital has been studied (V) and this drug was found to be less effective than phenobarbital and with an anticonvulsant effect which was slower in onset. The effect seemed, however, to be more sustained than that of phenobarbital and the difference observed may perhaps be explained by a conversion of the benzoyl analogues to phenobarbital. However, the toxicity was lower for the latter drug.

3. Ectylurea

Ectylurea was stated in the literature to lack anticonvulsant activity, which seemed to be somewhat surprising in view of the chemical relation to phenylacetylurea (phenacemide). This latter compound has been found to be active both in animal tests and in epilepsy (references cited in Paper VI). We found, however, that the high-melting isomer (ectylurea) was effective in preventing convulsions induced by strychnine and picrotoxin (VI). Furthermore, it was effective against electroshock and the effect was rapid in onset. A dose of about twice the ED_{50} value gave effects which subsided after three hours. The low-melting isomer showed, however, no anticonvulsant activity. In large doses it even produced convulsions, which could be prevented by its own isomer (ectylurea). At a dose of 250 mg/kg orally ectylurea showed an increase in the tolerance to strychnine- and picrotoxin-induced convulsions comparable to that of phenobarbital sodium at a dose of 25 mg/kg. However, this latter drug was found (Paper V) to be the most potent agent against electroshock and was about 85 times (equimolecular) more active than ectylurea. These differences in sensitivity to different convulsion stimuli could perhaps be explained by the fact that phenobarbital is less active against chemically-induced seizures. The oral toxicity of ectylurea could not be determined as the LD_{50} exceeded 4.5 g/kg.

As a rule the activity against electroshock increases when an alkyl group is replaced by phenyl [72]. This was actually done in ectylurea and two isomers of phenylcrotonylurea were consequently obtained, one high-melting (197°C) and one low-melting (185°C). The high-melting form showed anticonvulsant activity in the electroshock procedure at half the equimolecular doses of the alkyl compound (ectylurea) (Table 1, in V). As for this latter compound, the low-melting isomer showed no activity in doses up to 500 mg/kg.

The method introduced had furnished the possibility of repeating the tonic hindlimb extension in each animal without the tonic phase being changed. The fact that antiepileptic drugs are often used throughout

the entire lifetime favours the reflection that this method might be used in studying the development of tolerance to anticonvulsant drugs. Thus, as an example of this type of test, the effects of ectylurea were followed during an administration period of 5 weeks. The drug was administered orally and a persistant effect was found during the whole period (Fig. 5 in V).

4. Correlation to clinical potency

As has been pointed out for the analgesic drugs, the value of a comparison between clinical effectiveness and the activity in animals is limited. Close and Spielman [83] have stated that a simple and predictable relationship does not appear to exist between the results from animal experiments of anticonvulsants and clinical findings. However, drugs effective against electroshock have often been found to be effective against grand mal and psychomotoric epilepsy, while agents effective against chemoshocks have perhaps some, but often poor predictability against petit mal. With the method introduced there appeared to be a correlation between the effectiveness in mice and the clinical type of epilepsy. Grand mal and psychomotoric agents were most effective in mice, and petit mal drugs the least active. Paper V contained a speculation over a proposed indication of epilepsy for ectylurea and its phenyl analogue, and the preliminary results seemed to confirm the prediction of petit mal for the former [84].

5. Effects of antiepileptic drugs in immature animals

A log dose-response relationship were found in all age-groups after subcutaneous administration of phenytoin (VIII). The slopes were calculated and comparisons between the adjacent age groups were made. It was found that 5-day-old animals had a slope which differed significantly from that of 3- and 9-day-old mice. The slope for 18-day-old mice also differed significantly from that of 15-day-old mice.

The possibility thereby presented of following the duration of the anticonvulsive effects during maturation was utilized to study some well-known antiepileptic drugs (IX). Following drugs were investigated: Acetazolamide, dimethadione, ethosuximide, mephobarbital, phenobarbital sodium and trimethadione. Throughout the postnatal period a fixed dose of each drug was used; the dose being based on the ED_{50} values in adult animals and the knowledge of the subcutaneous ED_{50} value for pentylentetrazol seizures found by Ferngren [15, 16]. Furthermore, Ferngren has stressed that the body surface area as a principle for extrapolation of median doses of barbiturates from the adult to the neonatal mice could not be used.

In general, the drugs reduced the seizure duration significantly compared to the control animals, with some exceptions. Thus, the results indicated the existence of some critical age periods: Newborn, 9-day-old and 21-day-old mice, in which certain anticonvulsants had a poor effect, although they were active at other ages. As has been pointed out in Paper

IX, this could be a question of dosage. However, mephobarbital lacked effect in 9-day-old mice (Fig. 3 in IX) and ethosuximide in 21-day-old (Fig. 10 in IX). Acetazolamide in a massive dose of 1 g/kg showed a weak effect before the age of 12 days (Figs. 11 and 12 in IX). The found effects in the different age-groups supported the hypothesis that acetazolamide exerts its action via inhibition of brain carbonic anhydrase and accummulation of intracellular carbon dioxide (see references cited in Paper IX).

The duration of all anticonvulsant drugs tested except ethosuximide lasted more than 24 hours in the immediate newborn period. The lack of effect in 21-day-old mice of this latter drug is contrary to the findings in adults, but the difference could either be a consequence of the dosage used or a more rapid biotransformation in these younger animals.

In general, the prolonged action in the immediate newborn period could be a consequence of a slower metabolism and excretion of the drugs.

6. Conclusions

The reliability and the sensitivity of the method introduced have been investigated by testing 11 well-known antiepileptic drugs in adult animals. The drugs were administered orally and were found to be effective in preventing the tonic hindlimb extension in dose levels within a safe distance from toxic doses. The method seems to be sensitive since the ED_{50} values are low, 10—50 % throughout compared to those reported with other electroshock procedures for the same substances. Even petit mal agents such as ethosuximide and trimethadione were found to be effective. The possibility of following the time response curve of the anticonvulsive effect in each animal has *inter alia* given the results that, generally, the grand mal agents produce a slow onset of the anticonvulsant activity while drugs against psychomotoric and petit mal epilepsy showed a rapid onset.

The method has further been applied in investigations of potential anticonvulsants. Ectylurea was stated in the literature to lack anticonvulsant activity, but was found in the present investigations to be effective in preventing convulsions elicited by electroshock and chemical agents. The effect was even found to persist after prolonged administration during a period of 5 weeks. A phenyl substitution in ectylurea increased the effectiveness against electroshock twofold while a benzoyl substitution in phenobarbital decreased the activity ninefold.

The high-frequency electroshock procedure had furnished the opportunity of studying anticonvulsant activity in mice during postnatal development. The method was applied in studies of antiepileptic drugs given subcutaneously. Phenytoin was shown to give a dose-dependent reduction of the seizure duration in all age-groups. Six other antiepileptics given in single doses were generally effective in reducing seizure durations compared to control animals with some exceptions, which indicates that certain age-periods (newborn, 9-day-old and 21-day-old mice) are critical for the anticonvulsive effect.

V. Summary

Methods based upon a careful standardized stimulation with high-frequency electrical square waves, have been elaborated for quantitative and, to some extent, qualitative studies of analgesic and anticonvulsant activity in mice.

The sensitivity and reliability of the introduced methods were found to be quite satisfactory, and the procedures are well adapted for the screening of potential analgesics and anticonvulsants.

The new possibilities offered by the methods have been utilized for closer studies of certain drugs. Thus, the mechanism of the analgesic action of some of the salicylates has been investigated and the results have indicated that the action against the pain stimulus used seems to be of central origin.

The use of these procedures has given analgesic and anticonvulsant effects of drugs which earlier had been reported to be inactive in animals with other test methods *i. e.* analgesics as pentazocine, phenazone and salicylates resp. anticonvulsants as ectylurea, ethosuximide, sodium bromide and trimethadione.

The new possibility of following the duration of anticonvulsant activity in each intact animal has been utilized both in adult as in immature mice, and it was shown that certain ages are critical for the anticonvulsant effect as well as for the development of the seizure pattern, which could be induced already in newborn animals.

Acknowledgements

The present investigations have been carried out at the Department of Pharmacology and Toxicology of the Faculty of Pharmacy, Uppsala University, and at the Central Research and Control Laboratory of the Association of Swedish Pharmacies. I wish to express my sincere gratitude to the Head of the Department, Professor *Håkan Rydin* for his never-failing interest, useful advice and constant encouragement.

I am greatly indepted to Docent *Stellan Ljungberg* who followed my work from the initial stages and I am deeply grateful for his never-failing interest, useful advice and valuable suggestions throughout the work.

I am further indepted to my co-authors Dr. *Harry Ferngren*, Docent *Stellan Ljungberg*, Docent *Sven Ström*, Mr. *Uno Otto*, Dr. *Bengt Danielsson*, and Apotekare *Siv Johansson* for their friendly and willing cooperation.

My thanks are also due to my collegues at the Department for their generous help and I wish to express my particular appreciation to Miss *Margareta Helleday*, Miss *Inga-Lise Kinell*, Miss *Anna Lisa Paulsson*, Mrs. *Kristina Splinter* and Mrs. *Waltraut Szczerba* for skilful technical assistance. I further wish to thank

Miss *Eva Rönnerwall* and Mrs. *Pamela Zacharias* for linguistic and technical help in the preparation of the papers.

I offer my sincere thanks to the Association of Swedish Pharmacies for giving me the opportunity of carry out the investigations.

The work has been supported by grants from the Faculty of Pharmacy, which is gratefully acknowledged.

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Received April 8, 1969.

AB Realtryck, Stockholm 1969

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